

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problems Mailbox.**

From: Chen, Shin-Lin
Sent: Thursday, January 08, 2004 6:18 PM
To: STIC-ILL
Subject: articles 1632

Please provide the following articles. Thanks!
Serial No. 09/730,790.

L10 ANSWER 32 OF 38 MEDLINE on STN DUPLICATE 4
AN 96075561 MEDLINE
DN 96075561 PubMed ID: 7586219
TI VEGF165 expressed by a replication-deficient recombinant adenovirus vector induces angiogenesis in vivo.
AU Muhlihauser J; Merrill M J; Pili R; Maeda H; Bacic M; Bewig B; Passaniti A; Edwards N A; Crystal R G; Capogrossi M C
CS Pulmonary Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA.
SO CIRCULATION RESEARCH, (1995 Dec) 77 (6) 1077-86.

L10 ANSWER 30 OF 38 MEDLINE on STN DUPLICATE 3
AN 96290680 MEDLINE
DN 96290680 PubMed ID: 8730841
TI Liposome-mediated BDNF cDNA transfer in intact and injured rat brain.
AU Iwamoto Y; Yang K; Clifton G L; Hayes R L
CS Department of Neurosurgery, University of Texas Houston Health Science Center, Houston 77030, USA.
NC PO1 NS31998 (NINDS)
RO1 NS21458 (NINDS)
SO NEUROREPORT, (1996 Jan 31) 7 (2) 609-12.

L10 ANSWER 26 OF 38 SCISEARCH COPYRIGHT 2004 THOMSON ISI on STN
AN 1998:368339 SCISEARCH
GA The Genuine Article (R) Number: ZM121
TI Responses of young and aged rat CNS to partial cholinergic immunolesions and NGF treatment
AU Wortwein G; Yu J; ToliverKinsky T; PerezPolo J R (Reprint)
CS UNIV TEXAS, MED BRANCH, DEPT HUMAN BIOL CHEM & GENET, GALVESTON, TX 77555 (Reprint); UNIV TEXAS, MED BRANCH, DEPT HUMAN BIOL CHEM & GENET, GALVESTON, TX 77555; RIGSHOSP, LAB NEUROPSYCHIAT, DK-2100 COPENHAGEN, DENMARK
CYA USA; DENMARK
SO JOURNAL OF NEUROSCIENCE RESEARCH, (1 MAY 1998) Vol. 52, No. 3, pp. 322-333.

L10 ANSWER 24 OF 38 SCISEARCH COPYRIGHT 2004 THOMSON ISI on STN
AN 1999:135300 SCISEARCH
GA The Genuine Article (R) Number: 164UH
TI Nerve growth factor expressed in the medial septum following in vivo gene delivery using a recombinant adeno-associated viral vector protects cholinergic neurons from fimbria-fornix lesion-induced degeneration
AU Mandel R J (Reprint); Gage F H; Clevenger D G; Spratt S K; Snyder R O; Leff S E
CS LUND UNIV, WALLENBERG NEUROSCI CTR, NEUROBIOL SECT, SOLVEGATAN 17, S-22362 LUND, SWEDEN (Reprint); CELL GENESYS INC, DEPT PRECLIN BIOL, FOSTER CITY, CA 94404; SALK INST BIOL STUDIES, GENET LAB, LA JOLLA, CA 92037
CYA SWEDEN; USA
SO EXPERIMENTAL NEUROLOGY, (JAN 1999) Vol. 155, No. 1, pp. 59-64.

Shin-Lin Chen

We examined the temporal profile of the expression of brain-derived neurotrophic factor (BDNF) cDNA containing a viral promoter following the injection of liposome cDNA complexes into the intact and traumatically injured rat brain. *In situ* hybridization and PCR confirmed the presence of injected BDNF cDNA for at least 6 days after injection. A similar profile of BDNF cDNA was observed when it was injected following cortical impact injury. mRNA was also localized around the injection areas. These results suggest that liposome-mediated delivery of neurotrophin cDNA may be a practical gene transfer method for treating traumatic brain injury.

Key Words: Gene transfer; Liposome; BDNF; cDNA; Traumatic brain injury; *in vivo*; Rat

Liposome-mediated BDNF cDNA transfer in intact and injured rat brain

Yoshihiro Iwamoto, Keyi Yang,
Guy L. Clifton and Ronald L. Hayes^{CA}

Department of Neurosurgery, University of Texas Houston Health Science Center, 6431 Fannin Street, Suite 7.148, Houston, TX 77030, USA

^{CA}Corresponding Author

Introduction

Brain-derived neurotrophic factor (BDNF) is a neurotrophin present in the rat brain that has therapeutic potential for treating various types of injury to the central nervous system (CNS).¹⁻⁴ Enhancing the availability of BDNF following traumatic brain injury (TBI) may therefore prove to be useful therapy.

Although the administration of exogenous proteins has generated important biochemical information and has therapeutic potential, significant limitations imposed by protein degradation and by the blood brain barrier restrict the clinical utility of this approach.⁵ Gene transfer is an alternative way in which BDNF can be introduced into the CNS. Viral vectors have been widely studied as carriers of genes into the CNS,⁶⁻¹⁰ as have cationic liposomes. The latter technique has certain advantages, including simplicity and safety.¹¹⁻¹³ Liposome-mediated β -galactosidase (β -gal) gene transfer has been reported in the adult mouse brain.¹⁴ Our laboratory is conducting systematic studies of the potential use of cationic liposomes for gene transfection in the CNS. Optimal concentrations of liposomes for transfection of the β -gal gene in primary septo-hippocampal cultures have been determined,¹⁵ and we have confirmed the relatively long lasting, but not permanent expression of functional nerve growth factor (NGF) in primary septo-hippocampal cell cultures following liposome-mediated gene transfer.¹⁶ The therapeutic potential of liposome-mediated BDNF gene transfection has been shown by its ability to reduce injury-induced neurofilament loss in primary septo-hippocampal cell cultures.^{17,18} We are

now examining the potential for *in vivo* transfection of neurotrophin cDNA to treat TBI.

Several recent reports have described the transfer of foreign genes into the CNS *in vivo*. Most of those experiments employed stereotactic injections into the brain parenchyma to deliver the gene, regardless of the vectors used.^{6,8-10,14} However, little is known about the distribution or time course of injected cDNA or mRNA expression. Here we examined the temporal profile of BDNF cDNA injected into the traumatically injured as well as in the intact rat brain.

Materials and Methods

Preparation of plasmids and liposomes: Previous studies indicated that the cytomegalovirus (CMV) is a prudent initial choice of viral promoter. We used a pUC19-based plasmid containing a CMV promoter as an expression vector for BDNF transfection.¹⁹ Rat BDNF cDNA was subcloned into a unique *NotI* site under the control of the CMV promoter (pCMV/BDNF). This plasmid has been proved to transfect genes into the CNS cells.¹⁷ We used the commercially available DOTMA and DOPE (Gibco BRL) liposome formulated from a 1:1 (wt:wt) mixture of the cationic lipid *N*-[1-(2,3-dioleoyloxy)propyl]-*n,n,n*-trimethylammoniumchloride (DOTMA) and dioleoyl phosphatidylethanolamine (DOPE) in membrane-filtered water.

Stereotactic injection of cDNA: Male Sprague-Dawley rats (250-300 g, Harlan Laboratories) were anesthetized with sodium pentobarbital (50 mg kg⁻¹, i.p.) and their heads were fixed using a stereotactic

fracture. After exposure of the skull, a small burr hole was opened at the point 3.6 mm posterior to bregma and 1.8 mm lateral to the sagittal suture. A needle (30 G) was inserted to a depth of 3.8 mm and fixed. Purified plasmid DNA (8 μ g) was mixed with 20 μ l liposomes and either liposomes or plasmid DNA/liposomes was injected (1.2 μ g min⁻¹) stereotactically into the right dorsal hippocampus of intact rats. Rats were sacrificed 1 h, 1 day, 3 days or 6 days after injection.

In situ hybridization: We employed an *in situ* hybridization technique to locate regions of BDNF cDNA and mRNA. Rats were perfused intracardially with 120 ml saline at 40 ml min⁻¹, followed by 200 ml fixative A (0.8 g NaOH, 8 g paraformaldehyde, 1.64 g sodium acetate in 200 ml distilled H₂O, pH 6.5) at 20 ml min⁻¹, then fixative B (1.4 g NaOH, 14 g paraformaldehyde, 13.35 g borax, pH 9.5). Brains were cryoprotected in 10% sucrose/fixative B overnight at 4°C. Coronal slices (16 μ m) were prepared and mounted on slides. The *in situ* hybridization technique employed has been routinely used by our laboratory and has been described previously.²⁰⁻²² The samples were subjected to 0.001% proteinase K digestion at 37°C for 20 min, then immersed in 0.1 M triethanolamine with 0.25% acetic anhydride for 10 min. After dehydration, hybridization was performed with a ³³P-labeled BDNF cRNA probe (10⁷ c.p.m. ml⁻¹) at 60°C overnight. Antisense and sense cRNA probes were obtained from cDNA cloned in a pKS⁻ vector using T7 or T3 RNA polymerase. After hybridization, the slides were washed sequentially in 4 \times , 2 \times , 1 \times , 0.5 \times , 0.1 \times SSC and dehydrated. Brain slices were exposed to Kodak XAR-5 film. For RNA digestion, some samples were treated with 0.002% RNase A at 37°C for 30 min following proteinase K digestion.

Semi-quantitative PCR: Rats were decapitated under anesthesia (sodium pentobarbital, 70 mg kg⁻¹, i.p.) and the brains were removed. The right hippocampus was dissected at 4°C. Tissue DNA was isolated by digestion with proteinase K and phenol/chloroform/isoamyl alcohol extraction. For PCR, one pair of forward and backward primers of BDNF were used. The sequence of BDNF/5 primer was 5'-GCAAACATGTCTATGAGGGT-3', and of BDNF/3 was 5'-GGTCAGTGTACATACACAGG-3'.²³ Actin DNA was used as an internal control. Another pair of primers for actin was introduced into the BDNF PCR process. Actin and BDNF cDNA were co-amplified in the same tissue sample. Forty pmol of each primer and 1 μ g DNA were used for PCR. PCR was carried out in a programmable heating block (Perkin-Elmer, Norwalk, CT) using cycles consisting of denaturation at

95°C for 1 min, followed by annealing at 55°C for 1 min and DNA extension at 72°C for 2 min. After 30 cycles of PCR, samples were electrophoresed on 1.5% agarose gel. Gels were stained with ethidium bromide and photographed under u.v. light.

Production of traumatic brain injury: Male Sprague-Dawley rats (250–300 g, Harlan Laboratories) were anesthetized with sodium pentobarbital (50 mg kg⁻¹, i.p.). TBI was produced by a widely used, clinically relevant controlled cortical impact model described by Dixon *et al.*²⁴ Ten minutes after injury, 8 μ g of purified pCMV/BDNF plasmid DNA was mixed with 20 μ g of liposomes and either liposomes or plasmid DNA/liposomes were injected (1.2 μ g/min) stereotactically into the right hippocampus. *In situ* hybridization and semi-quantitative PCR analysis were performed as described above.

All animal studies conformed to the guidelines outlined in the Guide for the Care and Use of Laboratory Animals from the U.S. Department of Health and Human Services, and were approved in advance by the University of Texas Health Science Center at Houston Animal Welfare Committee.

Results

In situ hybridization after injection of BDNF cDNA in the intact brain (Fig. 1): In control rats injected with liposomes not complexed with cDNA, hybridization signals corresponding to antisense BDNF mRNA were detected in both the hippocampus and neocortex, though the sense probe for BDNF did not hybridize. In rats injected with BDNF cDNA, positive signals were detected in the hippocampus after hybridization with both antisense and sense BDNF cRNA probes 3 days and 6 days postinjection. These signals indicated the presence of both BDNF DNA and mRNA. To confirm the presence of DNA, mRNA was digested with RNase A before hybridization. We detected a strong signal, which indicated the presence of DNA after RNA digestion. We did not, however, see a significant DNA signal in sections from rats injected only with liposomes (data not shown). The BDNF cDNA signal extended beyond the injection site. Distribution of BDNF cDNA narrowed gradually, but still persisted 6 days postinjection. We attempted to digest DNA in order to detect mRNA, but were unable to digest DNA while retaining RNA.

Semi-quantitative PCR after injection of BDNF cDNA in the intact brain (Fig. 2): Since it is difficult to measure cDNA levels accurately by *in situ* hybridization, we used a semi-quantitative PCR technique to measure the time course of BDNF cDNA in the hippocampus after injection. Levels of

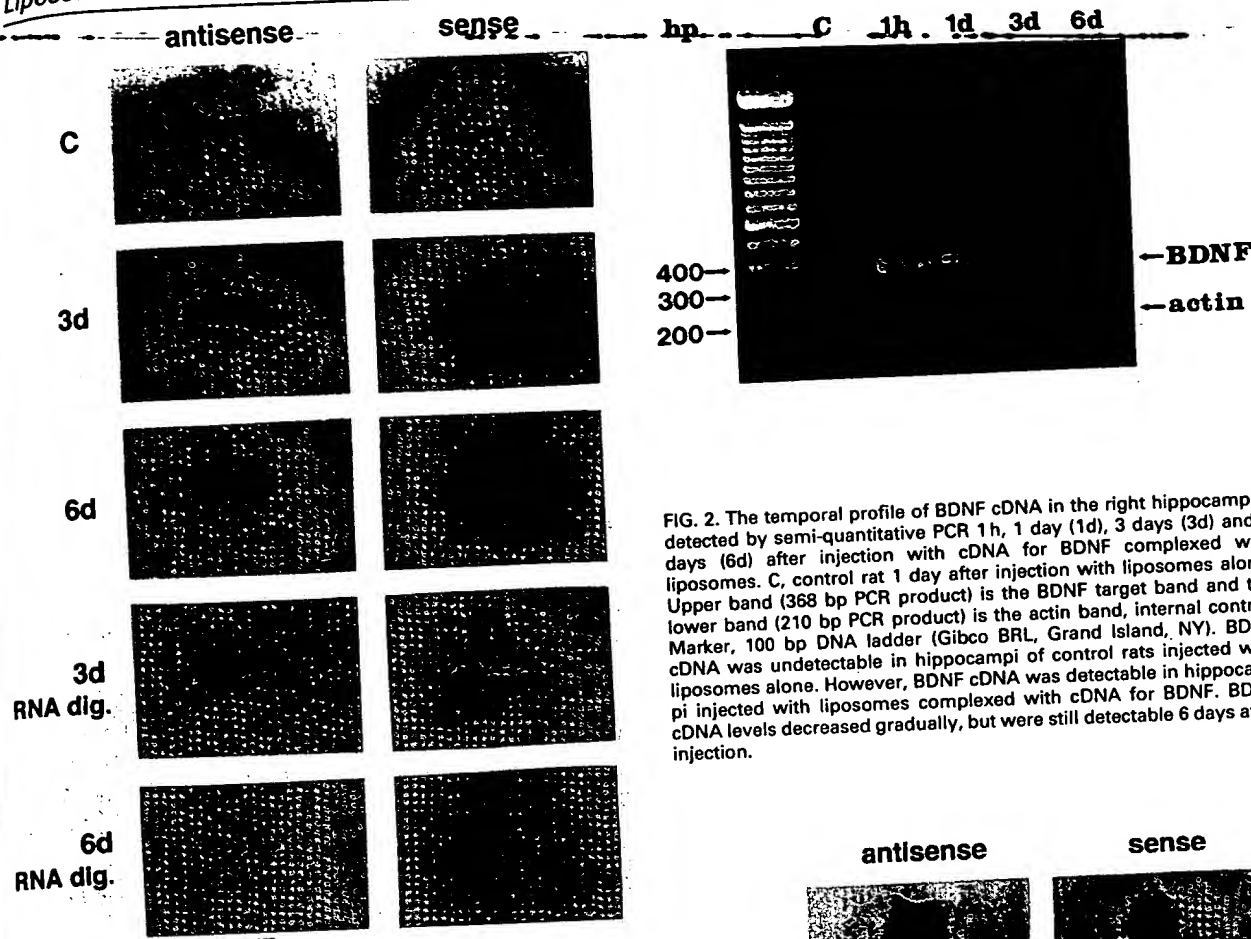


FIG. 1. Presence of BDNF cDNA in the rat brain following liposome-mediated BDNF cDNA transfer examined by *in situ* hybridization. C, control rat 1 day after injection with liposome alone. 3d, 6d, 3 days and 6 days after injection with liposomes complexed with cDNA for BDNF. RNA dig., hybridization following RNA digestion 3 days (3d) and 6 days (6d) after injection with liposomes complexed with cDNA for BDNF. The BDNF cDNA signal extended beyond the injection site. Distribution of BDNF cDNA narrowed gradually, but still persisted 6 days postinjection.

BDNF cDNA were undetectable in the hippocampi of control rats injected with liposomes alone. However, BDNF cDNA was present in hippocampi of rats injected with liposomes complexed with pCMV/BDNF. Maximal levels of BDNF cDNA were detected 1 h after injection. BDNF cDNA levels decreased gradually, but were still detectable 6 days after injection.

In situ hybridization (Fig. 3) and semi-quantitative PCR (Fig. 4) after injection of BDNF cDNA in the traumatically injured brain: *In situ* hybridization with both antisense and sense BDNF cRNA probes 1 day after cortical impact injury and BDNF cDNA injection showed the presence of both BDNF DNA and mRNA in the hippocampi. A strong signal in the right cortex with the antisense probe but not with the sense probe suggested the expression of BDNF mRNA following cortical impact injury.²² After

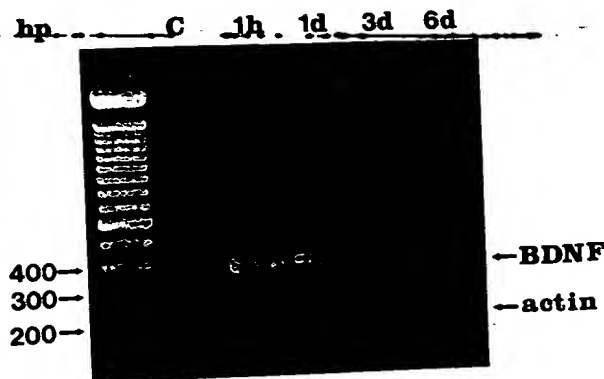


FIG. 2. The temporal profile of BDNF cDNA in the right hippocampus detected by semi-quantitative PCR 1 h, 1 day (1d), 3 days (3d) and 6 days (6d) after injection with cDNA for BDNF complexed with liposomes. C, control rat 1 day after injection with liposomes alone. Upper band (368 bp PCR product) is the BDNF target band and the lower band (210 bp PCR product) is the actin band, internal control. Marker, 100 bp DNA ladder (Gibco BRL, Grand Island, NY). BDNF cDNA was undetectable in hippocampi of control rats injected with liposomes alone. However, BDNF cDNA was detectable in hippocampi injected with liposomes complexed with cDNA for BDNF. BDNF cDNA levels decreased gradually, but were still detectable 6 days after injection.

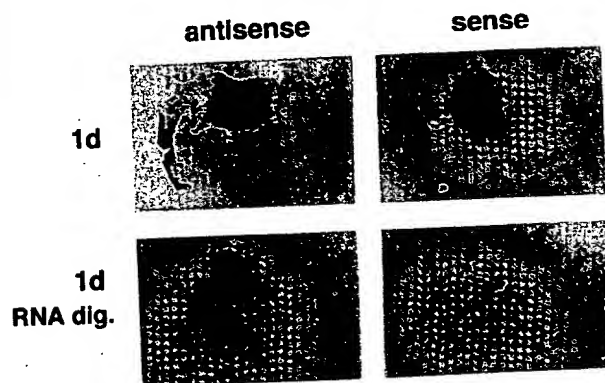


FIG. 3. Presence of BDNF cDNA in the rat brain 1 day (1d) after cortical impact injury and liposome-mediated BDNF cDNA transfer examined by *in situ* hybridization. RNA dig., hybridization following RNA digestion 1 day (1d) after cortical impact injury and injection with cDNA for BDNF complexed with liposomes. The BDNF cDNA signal was detected in the hippocampus. BDNF mRNA was also detected in the right cortex, probably reflecting endogenous BDNF mRNA expression induced by injury.

RNA digestion, a DNA signal remained, although the mRNA signal disappeared. PCR showed an undetectable level of BDNF cDNA in hippocampi of control rats injected with liposomes alone. However, BDNF cDNA was detected in hippocampi of rats injected with liposome complexed with pCMV/BDNF. Maximal levels of BDNF cDNA were detected 1 h after injection. BDNF cDNA levels decreased gradually, but were still detectable 6 days postinjection.

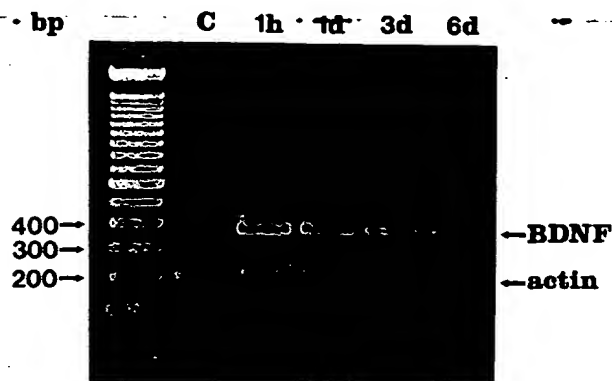


FIG. 4. The temporal profile of BDNF cDNA in the right hippocampus detected by semi-quantitative PCR 1 h, 1 day (1d), 3 days (3d) and 6 days (6d) after cortical impact injury and injection with cDNA for BDNF complexed with liposomes. C, control rat 1 day after injection with liposomes alone. Upper band (368 bp PCR product) is the BDNF target band and the lower band (210 bp PCR product) is the actin band, internal control. Marker, 100 bp DNA ladder (Gibco BRL, Grand Island, NY). BDNF cDNA was undetectable in hippocampi of control rats injected with liposomes alone. However, BDNF cDNA was detectable in hippocampi injected with liposomes complexed with cDNA for BDNF. BDNF cDNA levels decreased gradually, but were still detectable 6 days after injection.

Discussion

BDNF has a neurotrophic effect and therapeutic potential for treating various types of CNS injury.¹⁻⁴ Previous studies showed that the hippocampus is more vulnerable to TBI than is the neocortex.²⁵ BDNF mRNA increased only transiently in the ipsilateral cortex and bilateral hippocampus after cortical impact injury.²² Thus, maintaining a high level of BDNF in the hippocampus by gene transfer may be therapeutically beneficial.

We have therefore used *in situ* hybridization and PCR to study the temporal profile of BDNF cDNA and mRNA expression following liposome-mediated gene transfection. To our knowledge, this is the first report of the temporal profile of cDNA and mRNA injected into intact and traumatically injured rat brains. Injected BDNF cDNA and mRNA extended beyond the injection site and persisted for at least 6 days postinjection. These findings were observed in the traumatically injured brain as well as in the intact brain. Since foreign cDNA outside of cells is supposed to be destroyed within a few days, these data suggest that cDNA injected by our method is taken into CNS cells. Using unilateral intrahippocampal injections of a liposome-mediated pCMV/

BDNA expression vector, we also detected the signal contralateral to the site of injection suggesting, that liposome/vector complexes diffused into the contralateral hippocampal zones.

Although previous studies reported the preferential vulnerability of the hippocampus to TBI,²⁵ our data suggest that even injured hippocampal cells may incorporate neurotrophic cDNA. Because the presence of cDNA in cells is essential for the expression of protein following liposome-mediated gene transfer, expression of protein by our method can be expected. We are currently investigating protein expression following liposome-mediated neurotrophic gene transfer in intact and traumatically injured rat brains.

Conclusion

The present data demonstrate that liposome-mediated BDNF cDNA injected into the intact and traumatically injured rat brain persists for at least 6 days postinjection as assessed by *in situ* hybridization and PCR. These results suggest that liposome-mediated delivery of neurotrophin cDNA may be a practical method for transferring genes to treat traumatic brain injury.

References

- Cheng B and Mattson MP. *Brain Res* 640, 56-67 (1994).
- Nakao N, Kokaia Z, Odin P et al. *Exp Neurol* 131, 1-10 (1995).
- Friedman B, Kleinfeld D, Ip NY et al. *J Neurosci* 15, 1044-1056 (1995).
- Barinaga M. *Science* 264, 772-774 (1994).
- Barinaga M. *Science* 264, 773 (1994).
- Fink DJ, Sternberg LR, Weber PC et al. *Human Gene Ther* 3, 11-19 (1992).
- Culver KW, Ram Z, Wallbridge S et al. *Science* 256, 1550-1552 (1992).
- Davidson BL, Allen ED, Kozarsky KF et al. *Nature Genet* 3, 219-223 (1993).
- Kato K, Yoneda Y, Okada Y et al. *Mol Brain Res* 25, 359-363 (1994).
- Le Gal S, Robert JJ, Barrard S et al. *Science* 259, 988-990 (1993).
- Nabel EG, Gordon D, Yang ZY et al. *Human Gene Ther* 3, 649-656 (1992).
- Hug P and Sleight RG. *Biochem Biophys Acta* 1097, 1-17 (1991).
- Felgner PL, Gadek TR, Holm M et al. *Proc Natl Acad Sci USA* 84, 7413-7417 (1987).
- Roessler BJ and Davidson BL. *Neurosci Lett* 167, 5-10 (1994).
- Yang K, Faustinella F, Xue JJ et al. *Neurosci Lett* 182, 287-290 (1994).
- Yang K, Faustinella F, Xue JJ et al. *Neurosci Lett* 182, 291-294 (1994).
- Hayes RL, Yang K, Whitson JS et al. *Neurosci Lett* 191, 121-125 (1995).
- Whitson JS, Kampfl AW, Zhao X et al. *Brain Res* in press, (1995).
- McGregor GR and Caskey CT. *Nucleic Acids Res* 17, 2365 (1989).
- Simmons DM, Arriza JL and Swanson LW. *J Histochem* 12, 169-181 (1989).
- Yang K, Mu XS, Xue JJ et al. *Brain Res* 664, 141-147 (1994).
- Yang K, Mu XS, Yan HQ et al. *J Neurosci Res*, in press (1995).
- Giordano T, Pan JB, Casuto D et al. *Mol Brain Res* 16, 239-245 (1992).
- Dixon CE, Clifton GL, Lighthall JW et al. *J Neurosci Methods* 39, 253-262 (1991).
- Jenkins LW, Moszynski K, Lyeth BG et al. *Brain Res* 477, 211-224 (1989).

ACKNOWLEDGEMENTS: The authors wish to thank Dr Hong Qu Yan for his valuable suggestions and Drs Juan J. Xue-Bian, Xu-Sheng Mu and Shi-Jie Liu for their technical assistance. This work was supported by Grants PO1 NS31998 and RO1 NS21458 from the National Institutes of Health.

Received 18 October 1995;
accepted 29 November 1995

General Summary

BDNF is a neurotrophin that has therapeutic potential for treating central nervous system injuries. To examine the possibility of using liposome-mediated BDNF gene therapy in traumatic brain injury, we investigated the temporal profile of BDNF cDNA injected in intact and injured rat brains by *in situ* hybridization and PCR. The injected cDNA was detectable for at least 6 days postinjection in the injured as well as in the intact brain. These data suggest that liposome-mediated delivery of neurotrophin cDNA may be a practical method for treatment of traumatic brain injury.